Chapter 5

HAPLOIDS: DERIVATION AND EVALUATION

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I. INTRODUCTION

When this author was in graduate school in the late 1950s, plant breeders could only dream that one day it would be possible to examine the genetic information in gametes, select the desired ones, and then combine the selected gametes to form a desired hybrid. It was apparent that such technology could be very beneficial for more rapid improvement of wind-pollinated, highly self-infertile species such as tall fescue (*Festuca arundinacea*). Considerable progress has been made since then. This chapter will be devoted to the development and application of concepts that have led to the production of haploid plants from immature gametes.

Theories discussed by Haberlandt[1] provided an early step in the evolution of plant biotechnology. He reasoned that each somatic cell in a plant contains the same genetic code and that somatic cells should be capable of developing into whole plants, if given the appropriate surroundings. However, technology was not sufficiently advanced to test the theories during his scientific career. Later, pioneering studies of plant tissue and cell culture by Philip White, R. J. Gautheret, F. C. Steward, Folke Skoog, and others provided a practical beginning to the *in vitro* culture of plant tissue and the regeneration of plants from the cultured tissue.

A report by Guha and Maheshwari,[2] in 1964, demonstrated *in vitro* production of embryos from anthers of *Datura innoxia*. A few years later, the same investigators[3,4] reported further success in development of embryoids from pollen grains of *Datura*. Within the next several years, a number of scientists, most notably Nitsch and colleagues,[5-8] were successful in culturing haploid tobacco (*Nicotiana tabacum*) plants from immature anthers. A paper by Nitsch and Nitsch,[7] in 1969, was followed by an "explosion of interest" in this approach and in numerous attempts to culture androgenetic haploid plants from a wide range of species. It is of significance that the most success was obtained with tobacco and a few other members of the *Solanaceae* family, which includes *Datura*.

During the mid 1960s, this author began collaborative research with Reinert using cultured tobacco tissue to learn that phytochrome was present in dividing and recently divided cultured cells, and that phytochrome played a role in uptake and metabolism by such tissue.[9-11] The finding that red light (acting through the phytochrome system within the cultured tissue) favored certain metabolic responses in cultured tissue provided an important basis for selection of light and temperature conditions that were later used in development of our anther culture and plant regeneration procedures.[12-14] As success with tobacco anther culture evolved,[12-16] Buckner persuaded them to join with him in a collaborative approach in his tall fescue breeding program. Our goal was to accomplish with tall fescue what was already being done with tobacco. That is, to culture haploid plants from immature anthers, to evaluate the haploid plants under controlled environments and field stress conditions, and

to produce doubled haploids from the haploid plants that performed best in the evaluations.[17] Because tall fescue is wind pollinated and highly self-infertile, there is a high probability that each gamete (and gamete-derived haploid plant) may be genetically different from each other gamete (or haploid plant derived from another gamete). The remainder of this chapter is devoted to the development of theory and procedures that led to successful production and evaluation of cytologically verified haploid tall fescue plants from immature anthers.

II. DERIVATION OF HAPLOIDS

The objective was to cause the gametic genetic code to be expressed in the form of functional haploid tall fescue plants. After cloning such haploid plants via tillers or via tissue culture and plant regeneration (see Chapter 4 of this book), one should be able to evaluate them under a wide range of environmental conditions to identify the best genotypes. Chromosome numbers should then be doubled in the selected haploids to produce fertile pure lines with the characteristics that were selected during evaluation of the haploid plants. Derivation of tall fescue haploid plants can be approached by several different procedures.

A. Twin Seedlings

Haploid plants can occur as natural accidents. The occurrence of haploids via twin seedlings varies in frequency among genotypes, even within the same species. In a conventional forage grass breeding program, such haploid plants are usually lost because they are smaller than normal seed-borne plants and they are not fertile. However, they could be useful in study of chromosome pairing.[18]

B. Maternal Haploids

Maternal haploids have been derived in several crop plants. For example, haploid embryos develop on barley (*Hordeum vulgare*) plants after pollination by *H. bulbosum*.[19-22] After fertilization, the *H. bulbosum* chromosomes are eliminated during subsequent mitotic divisions, and the developing embryo is a haploid *H. vulgare*. Another example involves tobacco (*Nicotiana tabacum*) plants. The *N. tabacum* flowers are emasculated before pollen shed, and the stigmas are dusted with pollen from *N. africans* plants.[23] As with barley, the genetic contribution of the pollen parent is eliminated and gynogenetic haploid embryos develop.[23] Also, culture of some haploid plants from unfertilized ova should be possible, as is the culture of haploid plants from immature anthers.

Early experimental evidence suggests that gynogenetic haploids may be more vigorous than androgenetic haploids. This may be an important factor for future use of haploids in plant improvement. Certainly, comparisons of the two kinds of haploids should be made.

Because of the unique nature of the tobacco plant as a model system for comparison of the various approaches, it is possible to obtain anther-derived haploids, maternal haploids, and inbred seed from the same plant. To do this, (1) some floral buds can be excised to obtain immature anthers for culture, (2) some immature ova can be cultured, (3) anthers can be removed (before pollen shed) from other floral buds which are subsequently pollinated with pollen from *N. africans*, and (4) some of the flowers can be self-pollinated to produce inbred seed. Thus, the large numbers of microspores per anther, ova per flower, and flowers per plant should allow an investigator to obtain both androgenetic and gynogenetic haploids from the same tobacco plant. After doubling chromosome numbers in such haploids, the doubled haploids could be self-pollinated and progeny of the two kinds of doubled haploids could be compared with the inbred progeny of the same original source plant. Thus, tobacco can provide a valuable model system in which to compare the vigor and potential usefulness of anther-derived vs. maternal haploid plants of the same genotype. Currin and colleagues at Clemson University have initiated such studies.[24] These will undoubtedly provide an extremely useful model system for other crop plants, including tall fescue.

C. Anther-Derived Haploids

The pioneering research of Guha and Maheshwari[2-4] and that of Nitsch and Nitsch[7] led to considerable discussion of the potential value of anther-derived haploids in plant breeding.[15,25-27] High enthusiasm among plant breeders, geneticists, and administrators followed the early success with tobacco. However, culture of haploids from other species, especially the grasses, was much more difficult. For example, excised anthers had to be stimulated to first produce callus and then to regenerate plants from the callus in a two-step process with some cereal grasses including rice (*Oryza sativa*),[27] wheat (*Triticum aestivum*),[28] and barley.[29] Although some plants were regenerated from such callus, the total number was extremely low and frequently included albinos and various ploidy levels.[28] Using a modified approach, Wilson[30] obtained callus from barley spikes that were cultured in liquid medium which contained 10% sucrose. He regenerated about 40 plants from the callus, but only 2 of them were green and they soon died. Other investigators also found that callus which formed on high-sucrose media regenerated a higher percentage of albinos and other abnormal plants relative to callus formed on media with lower levels of sucrose.[31]

While early attempts to culture haploid grass plants from excised anthers were not very successful, some researchers were able to initiate and maintain callus cultures from somatic tissue of various members of the grass family.[32-27] Eventually, numerous green plants were regenerated from somatic tissue-derived callus cultures of forage grasses[34,35,37] (see also Chapter 4 of this book). Our attempts to obtain tall fescue anther-derived haploid plants via excised anthers and via anthers attached to a segment of panicle (nurse tissue) are discussed below.

1. Rationale and General Approach

Earlier experience with tobacco showed that it is possible to culture green plants from immature gametes in anthers, and that such plants have the same chromosome numbers as the gametes from which they originated.[5-8,14] The haploids have been useful for evaluation of gene expression in growing plants. Doubling the chromosome numbers of such haploids results in fertile, pure lines with the genetic characteristics that were selected in the original haploid plants.[13,15] These same capabilities with a highly heterozygous species such as tall fescue could be even more useful in accelerating development of superior breeding lines.

We used field-grown plants of a widely used cultivar, "Kentucky 31", as a source of tall fescue anthers. Field-grown source plants were preferred because they had demonstrated survival under field conditions. There were, however, some disadvantages to using field-grown plants as a source of anthers. Field-grown plants often contained an endophytic fungus (*Acremonium coenophialum*), and decontamination of the tissue was difficult. Tall fescue has the added disadvantage (relative to annuals such as tobacco, wheat, and barley) of being very seasonal. That is, this perennial species must go through a period of cold vernalization followed by long days to bring it into flower. A combination of the importance of tall fescue as a forage and turf grass, and the difficulties associated with its use in cell and tissue culture approaches, have resulted in a highly challenging area of research.

The tall fescue plants were selected (generally from vigorous field-grown plants in springtime) when the panicles showed about 2 to 4 cm above the flag leaf.[38] The plants were cut at the soil surface and immediately placed in bottles of tap water for transport to the laboratory. Clear plastic bags were then placed over the fescue panicles to avoid drying of the young tissue during the preculture period. The protected materials were placed in darkness at 5°C for various durations before the anthers were cultured. Beneficial effects of a "cold shock" treatment were discussed by Sunderland and Roberts.[39] In addition to possible benefits from the cold shock treatment, some flexibility in duration of storage time at 5°C allows collection of a large number of plants from field plots at the appropriate stage of development, with some flexibility to initiate the cultures. This flexibility allows more efficient use of laboratory facilities and personnel. We used cold storage durations ranging from 0 to more than 30 d before processing the materials and starting the cultures.

The first step in processing the material was to stage representative anthers and relate this to general appearance of the florets from which they came.[38] In this way, we were able to visually examine the panicles and to remove and discard spikelets with a high probability of having anthers with microspores that were either earlier or later than the uninucleate stage. The remaining portions of the panicles (with only the selected florets attached) were then surface decontaminated prior to culture of anthers. The panicles, with re-

maining spikelets, were immersed for 5 min in a 0.3% sodium hypochlorite solution to remove surface contamination. All remaining preculture steps were done with sterile instruments in a laminar-flow transfer hood. The panicles were then rinsed in three changes of sterile water to remove the sodium hypochlorite. No attempt was made to remove any contaminant that may have been present within the plant tissue because of the high probability of damaging the dividing plant cells. Both excised anther and anther-panicle cultures followed the same procedures from plant collection through surface decontamination of the selected portions of the panicles.

2. Excised Anthers

Anthers were excised from tall fescue panicles that were pretreated in various ways and they were cultured on various media that were suggested by previous investigators.[28,29,31,37] The basic medium was a slight revision of that used in the mid 1960s for tobacco.[10] It was modified from Linsmaier and Skoog[40] and from Murashige and Skoog.[41] The basic medium contained the inorganic salts and organic substances outlined in Chapter 4 of this book. Variables in culture media included 2 to 12% sucrose, 0.to 20 mg of 2,4-D per liter, 0 to 2 mg kinetin per liter, 0.0 to 2.5 g active charcoal per liter, and different levels of casein hydrolysate and inositol. Attempts were made to obtain haploid plants or callus from excised anthers of tall fescue plants of several cultivars and ecotypes from two different growing seasons.

a. Observations

No haploid plants or calli were obtained from any of the thousands of excised anthers in the initial studies with tall fescue. Even though all attempts with excised anthers failed, some of the same medium combinations and plant ecotypes were successful in development of callus from soft, young somatic tissue that was excised from the lower ends of peduncles and internodes. The results obtained with the young somatic tissue explants proved that the culture medium was capable of initiating and supporting callus growth from tall fescue. Nevertheless, fully expanded leaf and stem tissue from the same plants failed to form callus or to regenerate plants.

b. Interpretation

Several important concepts evolved from the combination of experiments with excised anthers, young somatic tissue, and fully expanded somatic tissue. It was apparent that the young stem tissue which contained many dividing and recently divided cells could continue to undergo cell division to form callus and regenerate plants,[37] whereas fully expanded somatic tissue would not form callus or plantlets when using our media and culture protocols.[37] It was also evident that the young somatic tissue which contained dividing or recently divided cells continued to undergo divisions to initiate callus and regenerate plantlets, while no callus initiation or plant regeneration occurred

from the excised anthers, which also contained dividing cells at the time of excision. There were several possible interpretations for these apparently conflicting responses. Perhaps the young stem explants contained some growth factor, or factors, that was needed for continued cell division to occur on the media under the culture conditions used. If true, perhaps the factor was missing, or present in inadequate amounts, in the excised anthers (this might be visualized as a need for a "critical mass" of tissue).

3. Anther-Panicle Nurse Culture

a. Theory

The relative ease of initiating callus formation and plant regeneration from young somatic tissue explants, and the complete failure with excised anthers from the same plant and on the same medium formulations, led to the hypothesis that suitable nurse tissue might be needed to provide a stimulus to the microspores in the cultured anthers. Several different approaches to the use of nurse tissue and/or the addition of plant extracts to the culture medium were considered. The desired goal was to cause the microspores within the anthers to continue divisions and to then develop into haploid plants. To accomplish this, the nurse tissue and/or culture medium should contribute a stimulus to the dividing microspores, but the nurse tissue should not form any callus or regenerate any plants that would appear to be similar to those originating from the microspores within the anthers.

Our previous work with tall fescue and its hybrids (Chapter 4 of this book) provided the needed starting point for the approach with nurse tissue. The key point in the earlier study was that young somatic tissue which contained dividing or recently divided cells would continue to undergo cell division to form callus and regenerate plants on the media used, whereas fully expanded leaf and stem explants from the very same plants remained green in culture, but failed to develop any callus or regenerate any plants. This led to the hypothesis that fully expanded panicle tissue might be successful as nurse tissue if it remained attached to the appropriate stage of anthers. Such a combination would allow the dividing microspores in the anthers to remain attached to their natural nutrient source while also being affected by the components of the culture medium. Also, if the panicles were carefully selected and trimmed so that all somatic tissue capable of cell division (on the selected culture medium) was removed, there should be no callus formation or plant regeneration from somatic tissue, while callus and/or plantlets would develop from the immature gametes.

b. Approach

Vigorously growing plants were preselected for desirable agronomic characteristics, forage quality factors, and winterhardiness. Plant materials were collected from the field at about 0900 to 1000 h when the panicles began to show above the flag leaf. At that stage of panicle development, there was a wide range of anther stages on each panicle.

As discussed under Section II.C.1, above, the tall fescue stems with emerging panicles were covered with plastic bags to maintain humidity and to avoid drying during storage at 5°C prior to preparation for culture. This allowed us to collect many inflorescences from field plants over a relatively short time period when plants were at the appropriate stage of growth and to then store them for up to several weeks prior to processing. We used cold storage preculture periods ranging from 0 to more than 30 d in the various experiments.

Special care was taken to avoid drying of the anthers during processing and placement on culture medium. This was accomplished by keeping some sterile water over the anthers in the dissecting dish while they (or small panicle segments) were handled prior to placement on the appropriate culture media. After a few preliminary experiments (discussed under Section II.C.3.c, below), preparation of panicles for the anther-panicle culture approach involved (1) identification and removal of florets with anthers having a high probability of being younger than the uninucleate stage (based on microscopic examination of similar material), and (2) removal of all panicle tissue from which the young florets were already removed. The desired goal was to remove all somatic tissue capable of callus initiation and/or plantlet regeneration under the culture conditions. If totally succesful, the somatic tissue would serve as nurse tissue and only the immature gametes would undergo cell divisions and develop into plants.

Nutrient medium composition is very important and should be carefully planned to give desired results. For example, the exogenous hormone balance should be such that it favors cell divisions followed by haploid plantlet regeneration. Use of a multiple-step approach seems logical. Our experience with various tissue culture approaches has shown that preliminary experiments are often useful to determine genotype responsiveness to the level of auxin in the medium. While high amounts of 2,4-D will frequently cause initiation of more callus, lower amounts result in a much lower frequency of abnormal callus and a lower frequency of abnormal plants among those regenerated from the callus tissue.

Tall fescue appears to be autonomous for cytokinin. That is, the balance between callus initiation and plant regeneration can be manipulated by merely adjusting the auxin level in the medium.[37] This is an important difference from tobacco in which the balance between callus growth and plant regeneration is manipulated by altering the exogenous auxin/cytokinin ratio. Tobacco callus can be caused to regenerate plants by decreasing the auxin/cytokinin ratio. This can be accomplished by either decreasing the auxin and leaving cytokinin constant or by increasing the cytokinin and leaving auxin constant.[16] With tall fescue and some other plants that are autonomous for cytokinin, a concentration of about 2 mg of 2,4-D per liter of culture medium is usually adequate to initiate callus formation from young somatic tissue.[37] Decreasing the concentration of 2,4-D to about 0.25 mg per liter, or even

less, then has the effect of decreasing the auxin/cytokinin ratio, which results in a decrease in new callus formation and an increase in plant regeneration from the callus.

Sugar is another medium component that can influence plant regeneration from callus.[29,30] With tall fescue somatic tissue, we have found that sucrose concentrations of 10 to 12% result in regeneration of more plants than are obtained with 2 to 3% concentrations. However, the greater number of regenerated plants associated with the higher sucrose concentrations in the medium also includes higher percentages of abnormal plants such as albinos.

To be successful with the tall fescue anther-panicle approach requires (1) very careful removal of all undesired ("young") somatic tissue, (2) care to avoid drying the fragile young anthers, and (3) use of suitable media with carefully controlled manipulation of the auxin level to first initiate embyrogenic callus from the microspores and to then regenerate haploid plantlets. The success of some of these manipulations can be influenced by factors as subtle as medium preparation and relatively minor temperature fluctuations during culture. Our earlier studies of phytochrome involvement in uptake and metabolism in tobacco callus[10] suggest that the light conditions of the culture room might be expected to influence responses to hormone level in the medium.

As those familiar with tissue culture and plant regeneration realize, there is frequently a fine line between success and failure. Therefore, one should first do some preliminary experiments to become familiar with the materials at hand and then take all precautions to select or develop protocols with the highest probability of success.

c. Results and Discussion

Our experiments with anther-panicle culture evolved through several stages. The preliminary attempts involved merely surface decontamination of the panicles, cutting them into 2- to 3-cm segments and placing them in petri dishes on the culture medium previously used successfully for "young" stem tissue,[37] and unsuccessfully for excised anthers of tall fescue.[38] No segments of the panicles were discarded prior to culture; therefore, some segments contained "young" somatic tissue, others only "aged" tissue, and still others were a mixture. The first visually detectable changes in the cultured panicle segments occurred in about 10 d. Unfortunately, it soon became apparent that callus formation was originating from young somatic tissue in some of the panicle pieces. On the other hand, these preliminary experiments offered encouragement because some of the segments appeared to initiate callus only from anthers and not from the attached somatic tissue. Other segments (from the older portions of panicles) did not produce any callus from either anthers or panicle tissue. The preliminary experiment was terminated when it became apparent that callus was forming on some of the "young" panicle tissue.

The interpretation of the preliminary anther-panicle experiment was that:

(1) the culture medium was suitable to initiate callus, (2) young somatic tissue (that capable of callus formation) should be removed before culture, and (3) a two-stage procedure should be used to culture the appropriate stage of anthers while attached to nurse tissue that was not capable of callus formation. The goal was to initiate embryogenic callus from only the microspore cells, and then transfer to fresh medium with a lower 2,4-D concentration to regenerate plantlets from the newly initiated callus.

The first attempt with anther-panicle culture following the interpretation outlined above resulted in successful derivation of haploid plants. Field-grown "Kentucky 31" tall fescue plants were collected when the panicles showed about 3 cm above the flag leaf. After transfer to the laboratory, the shoots were covered with plastic bags and held in darkness at 5°C for more than 3 weeks, while other materials from the same collection were used in various preliminary experiments. Based on those preliminary experiments, all spikelets with anthers earlier or later than the uninucleate stage were discarded (stage of anthers was estimated by visual appearance based on cytological examinations of similar materials). We also discarded the panicle segments from which young anthers were already removed. The panicles, with remainig spikelets, were then surface decontaminated for 5 min in 0.3% sodium hypochlorite. The decontaminated panicles were then rinsed in three changes of sterile water to remove the decontamination solution. Each panicle was then placed in a separate petri dish containing some sterile water to keep the anthers from drying during the preculture preparation process. The panicles were then cut into 2- to 3-cm segments and placed in petri dishes on appropriate culture media for the first stage. We used petri dishes because they were convenient to handle and to stack during culture. This was especially convenient if some of the panicle segments had to be "rescued" because others in the same dish contained an endophytic contaminant that became apparent during culture. The culture room was kept at 22 ± 1°C in darkness, except for observation periods that varied from several times per week to several times per day. It should be noted that such a fequency of exposure to low-intensity light could influence involvement of the phytochrome system, without introducing photodegradation of media components as could occur with prolonged exposure to intense light.

The initial culture medium was modified from the one used earlier for tobacco,[10] but with the auxin content used for tall fescue callus initiation as shown in Chapter 4 of this book. Details are reported here for convenience. Each liter of medium contained *inorganic salts*: 1.65 g NH_4NO_3, 1.90 g KNO_3, 0.44 g $CaCl_2 \cdot 2\ H_2O$, 0.37 g $MgSO_4 \cdot 7\ H_2O$, 0.17 g KH_2PO_4, 15.9 mg $Fe_2(SO_4)_3$, 32.0 mg EDTA, 6.2 mg H_3BO_3, 22.3 mg $MnSO_4 \cdot H_2O$, 8.6 mg $ZnSO_4 \cdot 7\ H_2O$, 0.83 mg KI, 0.25 mg $Na_2MoO_4 \cdot 2\ H_2O$, 0.025 mg $CoCl_2 \cdot 6\ H_2O$, and 0.025 mg $CuSO_4 \cdot 5\ H_2O$); *organic substances:* 100 mg *myo*-inositol, 0.1 mg thiamine·HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine·HCl, 2.0 mg glycine, 20 g sucrose, and 6 g agar; 2 mg 2,4-dichlorophenoxyacetic acid

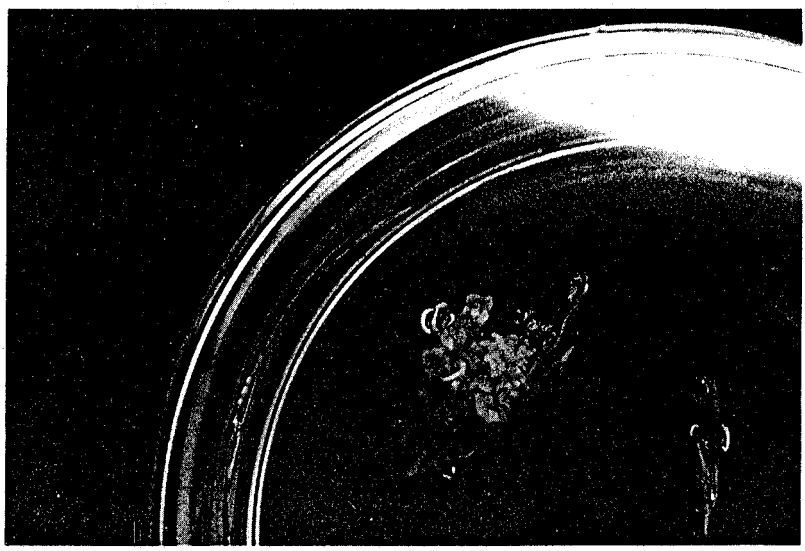

FIGURE 1. Plantlets and loose proliferation of cells from second stage of anther-panicle cultures of tall fescue. (From Kasperbauer, M. J., Buckner, R. C., and Springer, W. D., *Crop Sci.*, 20, 103, 1980.)

(2,4-D) was used for the first stage of the anther-panicle cultures. All components except sucrose, agar, and inositol were prepared as stock solutions, as described in Chapter 4 of this book. The pH was adjusted to 5.7 with 2 *N* NaOH after all components were added and the agar was melted. All media were autoclaved at 121°C for 15 min. About 50 ml of medium was added to each 100 × 20-mm sterile plastic petri dish and allowed to cool and stand for at least 1 d before use.

The second-stage medium was the same as the first-stage except that it had a lower 2,4-D concentration (i.e., 0.25 mg of 2,4-D per liter of medium) in order to cause the newly initiated callus to regenerate plants. The objectives were to stimulate embryogenic callus initiation from the uninucleate stage cells in the immature anthers during the first stage, and to then cause regeneration of haploid plants during the second stage. In this experiment, the materials were transferred from the first-stage medium (callus initiation) to the second-stage medium (plant regeneration) soon after the loose proliferation of cells became visually detectable.

A loose proliferation of cells with some plantlets emerging is shown in Figure 1. The first evidence of plantlet formation was a large number of tiny green flecks scattered through the proliferation of cells (because of the limited amount of material, none of it was sacrificed for microscopic study of embryoid formation). As plantlets began to develop, they were transferred to a

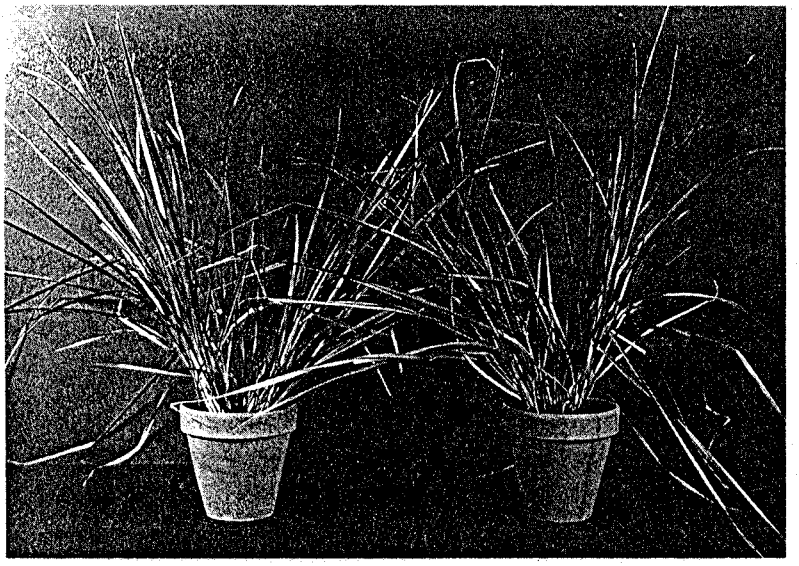

FIGURE 2. Haploid tall fescue plants that were derived via anther-panicle culture. (From Kasperbauer, M. J., Buckner, R. C., and Springer, W. D., *Crop Sci.*, 20, 103, 1980.)

rooting medium (half strength of the same basic formulation, but with no 2,4-D, and with sucrose and agar concentrations of 10 and 5 g/l, respectively). There were no albinos among the regenerated plantlets. More than 30 green plantlets that originated from different areas of the proliferation of cells were transferred to the rooting medium and placed in a growth chamber at 22 ± 1°C with 14-h daily light periods from cool white fluorescent lamps at about 80 μmol m^{-2} s^{-1}. After the plantlets developed roots, they were transferred to potting soil in a 20°C growth chamber and covered with tranparent plastic to maintain humidity during plant establishment. After several days, the plastic canopy was removed for about an hour twice per day to condition the plants. After the conditioning period, the plants received 14-h light periods from cool white fluorescent lamps at fluence rates that were increased over several days to about 550 μmols m^{-2} s^{-1}. Twenty-three of the plants survived the transfer to the large growth chamber in which they grew rapidly. Two representative plants are shown in Figure 2.

The chromosome number of each of the plants was determined by examination of root and leaf meristem metaphases by Springer. Details are discussed in Chapter 7 of this book. Chromosome numbers in somatic metaphase cells showed that all but one of the plantlets had the haploid number of chromosomes, n = 21 (Table 1, Figure 3). Some pronounced phenotypic differences existed among the various haploid lines, which supported the

TABLE 1
Chromosome Numbers in Somatic Metaphase Cells from Shoot Tips of Tall Fescue Plantlets Derived by Anther-Panicle Culture

Plant	Cells counted	Chromosome no.
1	27	21
2	50	39
3	34	21
4	25	21
5	42	21
6	30	21
7	25	21
8	35	21
9	25	21
10	6	21
11	27	21
12	25	21
13	25	21
14	25	21
15	25	21
16	31	21
17	25	21
18	29	21
19	25	21
20	25	21
21	15	21
22	25	21
23	50	21

From Kasperbauer, M. J., Buckner, R. C., and Springer, W. D., *Crop Sci.*, 20, 103, 1980.

concept that the different haploid plants originated from different microspores from a heterozygous "parent" plant. After chromosome counts were completed, the plants were grown under 12-h days to encourage tillering (as discussed in Chapter 4 of this book). After cloning the various haploid lines, ramets were transplanted to replicated field plots to evaluate them for winterhardiness, growth, development, and selected forage characteristics. Phenotypically, the 22 haploid lines ranged from early flowering with little leaf growth to sparse flowering with abundant leaf growth.[38] Some comparisons are shown in Table 2. Sugar, total nitrogen, moisture, dry matter disappearance, neutral detergent fiber (NDF), and phosphorous levels were used to estimate quality in summer and fall forage. Significant differences among lines occurred for all analyses. For example, NDF and sugar levels ranged from 48 to 59%, and 5.0 to 7.8%, respectively, for the summer harvest.[42] After field evaluation of the haploid lines, some of them were selected for development of doubled haploid lines, as discussed in Chapter 6 of this book.

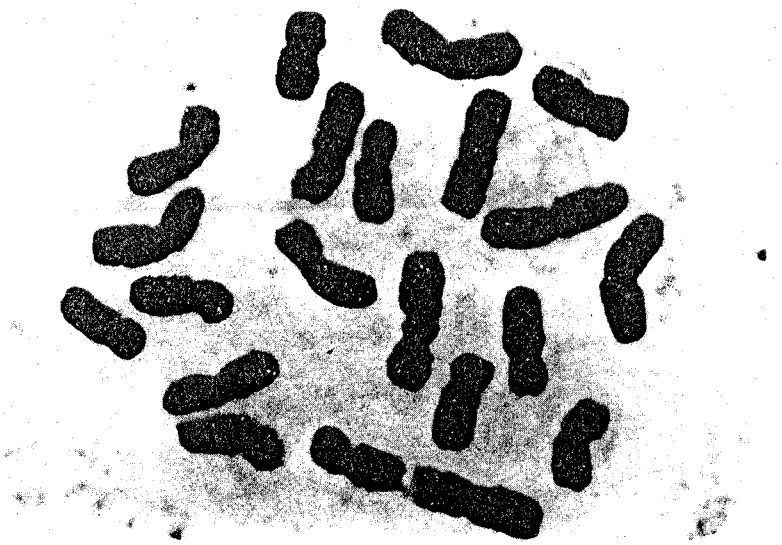

FIGURE 3. Chromosomes in shoot tip of a haploid tall fescue plant, n = 21. (From Kasperbauer, M. J., Buckner, R. C., and Springer, W. D., *Crop Sci.*, 20, 103, 1980.)

Subsequent experiments involving anther-panicle culture involved such variables as duration of preculture cold treatment, sucrose concentration of the medium, and 2,4-D levels during the first stage of anther-panicle culture. Plantlet regeneration was successful from a number of preculture cold treatments and culture medium combinations used in the first stage of culture. As suggested above, fewer abnormal plants were regenerated from anther-panicle segments that were cultured on the lower levels of 2,4-D and sucrose during the first stage. There were phenotypic variations among the normal-appearing regenerated plants, as would be expected among haploid plants that originated from different immature gametes of a wind-pollinated, self-infertile plant such as tall fescue. Because of the large number of regenerated plants and scheduling difficulties, cytological evaluations were limited to confirmation that randomly selected plants were haploids.

III. EVALUATION

A. Visual

Visual evaluation is suitable to identify and eliminate obviously abnormal plants. This can begin soon after plant regeneration by eliminating albinos and those that develop obviously abnormal roots and shoots. After establishment, additional visually detectable abnormalities may appear during greenhouse and field evaluations. Also, there may be visually detectable superior characteristics such as leafiness, color, and plant form.

TABLE 2
Relative Amounts of Vegetative and Reproductive Growth of the Different Androgenetic Haploid Lines Under Field Conditions near Lexington, KY, in 1980—1981

Haploid line[a]	Field growth: Veget. (22 July)	Field growth: Reprod.[b] (22 July)	Dry matter (g dry wt/5 plants): 25 Aug.	Dry matter (g dry wt/5 plants): 28 Oct.
1	8	6	54	100
3	2	10	15	27
4	6	6	41	55
5	10	3	55	91
6	8	4	59	77
7	8	1	51	75
8	8	4	52	84
9	9	4	52	92
10	9	4	59	101
11	10	4	76	104
12	6	6	26	44
13	2	4	7	7
14	9	3	58	134
15	10	3	60	111
16	10	4	58	132
17	4	6	18	31
18	10	5	69	126
19	8	6	66	111
20	10	4	59	111
21	1	10	—	—
22	9	6	71	103
23	8	5	52	92
LSD 0.05			16	32

[a] Haploid line numbers are those used in the paper by Kasperbauer et al.[38] Plant no. 2 was not a haploid; therefore, it is deleted from this table.

[b] Rating scale is 1 to 10. 1 = least, 10 = most.

From Kasperbauer, M. J. and Eizenga, G. C., *Crop Sci.*, 25, 1091, 1985.

B. Cytological

Cytological evaluation of putative haploids is an important part of the evaluation process. Confirmation of haploidy is needed before one attempts to develop doubled haploids. Details of the importance of and procedures for cytological evaluation of regenerated tall fescue plantlets are outlined by Springer in Chapter 7 of this book.

C. Field

Regenerated plants that advanced through preliminary visual and cytological evaluations can be increased through tillers for further detailed evaluation. After cloning the material, ramets can be used in replicated field (or controlled environment) studies to determine winterhardiness, drought tolerance, forage quantity, forage composition, disease resistance, etc. After field evaluation, selected haploid plants might be used in the development of fertile doubled haploid, pure lines with the same genetic code that was present in the "parent" haploid plant.[42]

IV. POTENTIAL SIGNIFICANCE

The experience with anther-panicle cultures clearly showed that it is possible to culture haploid plants from immature gametes of tall fescue. The haploid plants can easily be cloned via tillers or through cell culture and plant regeneration. Theoretically, use of the cloned haploid plants allows evaluation of the gametic genetic code under a wide range of field and other stress environments to identify those genetic combinations (haploid plants) that have the most desirable characteristics and to eliminate the undesirable ones. Doubling the chromosome numbers in the selected haploids should result in fertile pure lines that can then be used in hybrids or in synthetics. The doubled haploids could be crossed with other doubled haploids or they could be crossed with lines derived through conventional tall fescue breeding approaches. Another potential use of cytologically verified tall fescue haploids is as the female parent in the development of monosomic lines for basic genetic studies.[43]

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